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Actinobacteria Isolation from Forest Soils and Determination of Biological Activities

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Abstract: Actinobacteria bacterial group has one of the most populous population in microorganisms that extends very different and extensive habitats on earth especially the main character of the natural soil habitats. Since old times, bioactive metabolites of soil microorganisms have been studied and the results have provided that metabolites of these microorganisms have significant benefits to science, medicine, agriculture, and the pharmaceutical industry. In this study, isolation of Actinobacteria strains from forest soils, identification of morphological and molecular features, extraction of the bioactive metabolite of isolates and determination the antimicrobial, antibiofilm, and cytotoxic activities of bioactive extract were tested. The microbiological isolation methods for collected forest soil samples were used and after the determination of their morphological and molecular features, isolates were defined as *Brevibacterium spp.* that is a member of Actinobacteria. The antibiotic resistance of the isolates was determined by different methods and different concentrations of standard antibiotics. The chromium tolerance of isolates was also determined. The bioactive metabolites of isolates were produced in a modified medium and extracted. The antimicrobial, antibiofilm, cytotoxic activities of bioactive metabolites were determined against standard microorganisms and *Artemia salina* larvae were used as a test organism for cytotoxic tests. In the present study, results provided information about Actinobacteria that were isolated from forest soils. Isolates have antibiotic resistance and chromium tolerance abilities. Moreover, it has been shown that the Actinobacteria group is the largest bioactive metabolite producing group in terms of both antibacterial and antifungal activity and also contains a wide range of other compounds such as antibiofilm and cytotoxic compounds. The antimicrobial (MIC) concentrations of bioactive metabolites were detected 2500 µg/mL for standard bacteria cultures and 1250 µg/mL for yeasts. The antibiofilm (MBEC) value was determined at 1250 µg/mL. The 2500 µg/mL concentration of the extract was found to be the effective cytotoxic value. The results provide *Brevibacterium spp.* isolates have industrial and pharmaceutical potential and more detailed pharmaceutical researches are planned.

Keywords: Actinobacteria, *Brevibacterium*, antibacterial, antifungal

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INTRODUCTION

Soil is a highly complex, heterogeneous, and nutrient-limited environment consisting of an organic matrix with liquid and gaseous pores possessing the highest microbial diversity on the Earth (1). Molecular phylogenetic analyses reveal that the soil and rhizosphere can contain thousands of unique bacterial species per gram (2). The phylum of Actinobacteria is well-known for its ability to produce a wealth of natural products with structural complexity and with diverse biological activities (3). Actinobacteria bacterial group has one of the most populous populations of microorganisms that extend very different and extensive habitats on earth. Actinobacteria and its members are the main

characteristics of natural soil habitats. Especially, forest soils are eligible habitats for soil microorganisms, it contents organic-inorganic materials, various organisms, and their wastes. Actinobacteria are *Gram*-positive, free-living, saprophytic bacteria, widely distributed in soil, water, and colonizing plants with a filamentous structure.

Bacteria produce a vast array of secondary metabolites, which have diverse and important ecological functions. It has become increasingly clear that secondary metabolite production often is triggered by intra- and inter-specific interactions between soil bacteria. Soil bacteria produce a large number of secondary metabolites that have many different physicochemical and biological properties (1). Bioactive metabolites of microorganisms especially soil microorganisms have studied for many years and the results have provided that metabolites of soil microorganisms have significant benefits to science, medicine, agriculture, and the pharmaceutical industry. Actinobacteria have also ability to synthesize antiviral (4), antibacterial, antifungal (5), antibiofilm, antitumoral (6), insecticidal (7), antioxidative (8), anti-inflammatory (9), anti-biofouling (10), immunosuppressive (11), anti-parasitic (7), plant-growth-promoting and herbicidal compounds (12), enzyme inhibitors (6) and industrially important enzymes. From the 22,500 biologically active compounds that have been obtained from microbes, 45% are produced by Actinobacteria, 38% by fungi, and 17% by unicellular bacteria (13).

The main focus of this study was to isolate, characterize and identification of Actinobacteria strains that were collected from forest soils in the Çanakkale-Gelibolu region in Turkey. The isolated Actinobacteria strain was growth in special media and extracted to obtain the bioactive metabolite in crude extract form. The collected crude extract was evaluated with its antibacterial, antifungal, antibiofilm, and cytotoxic features.

EXPERIMENTAL SECTION

Isolation and characterization

The soil samples were collected from 6 to 10 cm depth from forest regions in Çanakkale-Gelibolu, Turkey. For sampling, moist and fertile soils that are rich in organic matter were selected.

After collection, soil samples were serially diluted up to 10^{-5} and inoculated to special isolation modified media. The content of this medium was as follows: Dextrose (4 g/L), yeast extract (4 g/L), malt extract (10 g/L), $CaCO_3$ (calcium carbonate) (2 g/L) and agar (4 g/L). The cultures were incubated at 37 °C for 3-5 days (14). This culture-dependent isolation method was continued until the pure colonies shown on the agar surface typically resemble Actinobacteria. The morphological characterization of these colonies determined by using the *Gram* staining method.

Molecular identification

The selected pure cultures were identified by using their 16S rRNA (ribosomal RNA) sequence. After, DNA isolation PCR (Polymerase Chain Reaction) amplification methods were used. The 27F/1492R universal primers were used and 1450 base pairs of 16S rRNA were amplified by using PCR. Two primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were used. Sequencing was performed by using the EURX Gene Matrix Bacterial Genomic DNA Purification Kit. The PCR protocol used was as follows: 95 °C for 5 minutes, 56 °C for 40 seconds, 72 °C for 60 seconds, 35 cycles of denaturation at 95 °C for 40 seconds, annealing at 56 °C for 40 seconds, and elongation at 72 °C for 45 seconds. Amplification was followed by a final extension at 72 °C for 5 minutes. After the PCR reaction was completed, 10 µL of the obtained PCR product was electrophoresed on a 1% 1× Tris-acetate-EDTA agarose gel containing ethidium bromide. PCR products were produced for bidirectional sequence analysis by mixing with their respective forward and reverse primers. The analyses were carried out in duplicate with the Sanger sequencing device and the Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems). The 16S rRNA gene sequence was compared to a sequence in the public database using basic local alignment search tool (BLAST - Basic Local Alignment Search Tool) on the national center for biotechnology information (NCBI (National Center for Biotechnology Information)) website (www.ncbi.nlm.nih.gov). The homology of the 16S rRNA sequence of the isolate was analyzed by using BLAST software (15,16).

Determination of the antimicrobial resistance of isolates

Antibiotic resistance was determined by the disc diffusion method. The antibiotic resistance of Actinobacteria culture were determined both by disc diffusion and microbroth dilution methods against standard antibiotic discs; AmC-30 (amoxicilin+clavulanic Acid 30/10 µg), SAM-20 (ampicillin+sulbactam 10/10 µg), IPM-10 (imipenem 10 µg) B (bacitracin 0.004 µg), OFX-10 (ofloxacin 10 µg) and TEC-30 (teicoplanin 30 µg). 100 µL of Actinobacteria culture inoculated to MHA (Mueller Hinton Agar) and the standard antibiotic discs were put on the agar and incubated at 37°C, for 24 hours. After the incubation period, the inhibition zones around the discs were measured (17). The microbroth dilution method (minimum inhibitory concentration - MIC) was defined as the lowest concentrations necessary for the inhibition of growth. 6 different concentrations (156.25, 78.125, 39.062, 19.53, 9.75, and 4.87 µg/mL) of standard antibiotics chloramphenicol, amoxicillin and ampicillin were used. Overnight microorganism culture was adjusted to McFarland's 0.5 standard. 100 µL of each antibiotic concentrations and 100 µL

of microorganism cultures were inoculated to the well plates and incubated at 37°C, for 24 hours. After the incubation period, the wells were stained by 20 µL resazurin dye to observe the color difference between dead and living cells.

Screening of the chromium tolerance

Potassium chromate (K_2CrO_4) and chromium(III) chloride ($CrCl_3$) salts were used at 100, 75, 50, 25, and 12.5 mg/100 mL concentrations. The chromium powders were added to PYE (peptone, yeast extract) and MHA (Mueller Hinton Agar) at proper concentrations by using the agar dilution method (18). The 50 µL of Actinobacteria culture was inoculated to the medium and incubated at 37°C, 96 hours. Microbroth dilution method was also used at 25, 12.5 6.25, 3.125, and 1.562 mg/100 mL concentrations. 100 µL of K_2CrO_4 and $CrCl_3$ was added to *Brevibacterium* sp. that adjusted to McFarland 0.5 and incubated at 37°C, for 24 hours. Following the incubation period, a 150 µL aliquot was taken from each concentration wells and inoculated to MHA and incubated at 37°C, 96 hours. After the incubation, colonies on the plate were counted.

Fermentation and extraction of bioactive metabolites from *Brevibacterium*

For the fermentation step, a submerged culture method was used. The pure *Brevibacterium spp.* isolate was grown in the modified medium containing; 4 g/L of glucose, 10 g/L of malt extract, 4 g/L of yeast extract, 1 g/L of K_2HPO_4 (potassium hydrogen phosphate), $MgSO_4$ (magnesium sulfate), NaCl (sodium chloride), 0.001 g/L of $FeSO_4$ (iron(II) sulfate) and 2 g/L of $CaCO_3$ (calcium carbonate). The prepared cultures were incubated 37°C, for 7 days. After the incubation period, organic solvent extraction was performed. The broth was filtered through a Whatman No.1 filter and cultures were extracted three times with equal volume of ethyl acetate (EtOAc) at a 1:1 ratio. Then the EtOAc solution was evaporated for dryness by using a rotary evaporator at 30°C, 80 rpm, under 187 mbar vacuum and the crude extract was collected (19).

Determination of minimum inhibitory concentration (MIC) of bioactive metabolite

5 different concentrations crude extract were applied to *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 29213, *Klebsiella pneumoniae* ATCC 700603, *Bacillus subtilis* NRRL B478, *Staphylococcus epidermidis* ATCC 14990, *Streptococcus pyogenes* ATCC 13615, *Listeria monocytogenes* ATCC 19111 and *Candida albicans* ATCC 90028 species by using 96 well plate assay. *Brevibacterium* crude extract was weighed and dissolved in %5 DMSO. Proper concentrations were 2500, 1250, 625, 312, and 156 µg/mL. Overnight microorganism cultures were adjusted to McFarland 0.5 standard. 100 µL of each extract concentrations and 100 µL of each microorganism cultures were inoculated to the well plates and incubated at 37 °C, for 24 hours. %5 DMSO, ketoconazole for fungi and chloramphenicol for bacteria species were also used as a control with 100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390, and 0.195 µg/mL values. After incubation period wells were stained by 20 µL resazurin dye to observe the color difference between dead and living cells (20, 21).

Biofilm eradication assay of bioactive metabolite

Microbial biofilms are communities of microorganisms embedded in a self-producing matrix, forming on living and nonliving surfaces. The inhibition effect of crude extract on biofilm formation was evaluated in 96-well plates. Briefly, overnight *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 14990, *Candida albicans* ATCC 90028 cultures were adjusted to McFarland 0.5 standard. 200 µL of each bacteria were inoculated to the well plates and incubated at 37 °C, for 48 hours to form the biofilm at the bottom of the wells. After the incubation period, wells were gently washed twice with 100 µL of 0.9% NaCl (physiological saline). 100 µL of concentrations (1250, 625, 312, 156, and 78 µg/mL) of crude extract were added to each well and incubated at 37°C, 24 hours. After the incubation period, wells were stained by 20 µL resazurin dye. (22, 23, 24)

Brine shrimp lethality test of bioactive metabolite

A 24-h LC_{50} (lethal concentration) bioassay was performed in a 96 well plate using nauplii of the Brine shrimp *Artemia salina* (25). Commercially sold *Artemia* mix was used. 18 g of the *Artemia* mix was poured to the 500 mL of distilled water and incubated 48-52 hours at 30 °C. After larvae had been seen, they were taken and counted. Brine shrimp lethality bioassay was determined with crude extract concentrations; 2500, 1250, 625, 312, and 156 µg/mL with 10 *Artemia salina* larvae in each concentration wells. After 24 hours incubation period, the alive larvae were counted.

RESULTS AND DISCUSSION

Isolation, characterization and molecular identification of Actinobacteria

In the present study, 25 Actinobacteria strains were isolated from collected soil samples. *The Gram* staining method was applied to all of these isolates. Some of them are shown in Figures below 1. a, b, c.

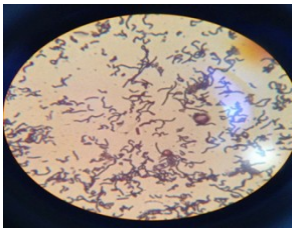

Figure 1.a

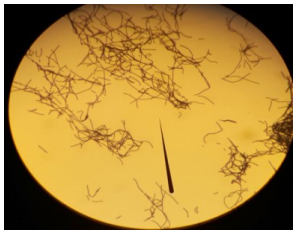

Figure 1.b

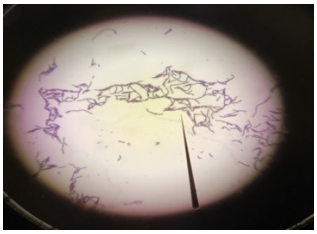

Figure 1.c.

Actinobacteria isolates after *the Gram* staining method.

The selected pure cultures were identified by using their 16S rRNA sequence. The results showed that most of them identified as *Brevibacterium spp.* BLAST analysis of the isolate showed that it is 100% similar to *Brevibacterium spp.* called *Brevibacterium frigoritolerans* (Accession Numbers: MN062932.1 and MN845150.1) which are the many *Brevibacterium* species available in the Gen Bank. *Brevibacterium* is one of the soil microbial communities that uncommon members of the Actinobacteria class. *Brevibacterium* is a member of the Actinobacteria phylum and genus of the Brevibacteriaceae family that is aerobic, non-sporulating, *Gram*-positive rod to coccoid shape and grows in a saline environment.

Determination of the antibiotic resistance of isolates

The antibiotic resistance of these isolates was determined by using standard antibiotic discs; AmC-30 (amoxicilin+clavulanic acid 30/10 µg), SAM-20 (ampicillin+sulbactam 10/10 µg), IPM-10 (imipenem 10 µg), B (bacitracin 0.004 µg), OFX-10 (ofloxacin 10 µg) and TEC-30 (teicoplanin 30 µg). It showed the most sensible reaction to IPM-10 (imipenem 10 µg). Also, the other values are shown in Table 1. Additionally, the MIC values are 78.125 µg/mL for chloramphenicol and 4.87 µg/mL for amoxicillin and ampicillin.

Table 1. Antibiotic resistance results from the disc diffusion method in terms of millimeters (mm).

Standard Antibiotic Discs	***Brevibacterium spp.* inhibition zone diameters (mm)**
AmC-30	19
SAM-20	16
IPM-10	20
TEC-30	8
OFX-10	12
B-0.04	0

Screening of the chromium tolerance

While 150-160 colonies were counted in chromium(III) chloride ($CrCl_3$) plate at 50 mg/100 mL concentration, 25 mg/100mL concentration plate formed 200-300 colonies. In 50 mg/100 mL potassium chromate (K_2CrO_4) plate 50-100 colonies and in 25 mg/100 mL plate 100-150 colonies were counted. Additionally, at the results of microbroth dilution for chromium(III) chloride ($CrCl_3$) and potassium chromate (K_2CrO_4), there was no formation of colonies. The test will be repeated using different concentrations. Chromium is one of the poisonous polluting metal ions that is found in soils. Several studies have demonstrated *Brevibacterium spp.* has chromium tolerance and chromium degradation abilities. Hence, chromium tolerance of isolates was tested by the agar dilution method. It showed significant tolerance to potassium chromate (K_2CrO_4) and chromium(III) chloride ($CrCl_3$) salts.

Determination of minimum inhibitory concentration (MIC) of bioactive metabolite

The MIC value of bioactive metabolite extract was tested at 2500, 1250, 625, 312, 156 µg/mL concentrations. The results are shown in Tables 2 and 3. The yeast species are more sensitive than bacteria species.

Table 2. MIC values of metabolite and chloramphenicol against bacteria species.

	MIC Values (µg/mL)	
Microorganisms	**Bioactive Metabolites**	**Chloramphenicol**
Escherichia coli ATCC 35218	2500	78.125
Staphylococcus aureus ATCC29213	2500	4.882
Listeria monocytogenes ATCC 19111	2500	9.765
Klebsiella pneumonia ATCC 700603	2500	19.531
Bacillus subtilis NRRL B478	2500	2.441
Staphylococcus epidermidis ATCC 14990	2500	9.765
Streptococcus pyogenes ATCC 13615	2500	39.0625

Table 3. MIC values of metabolites and ketoconazole against yeast species.

	MIC Values (µg/mL)	
Microorganisms	**Bioactive Metabolites**	**Ketoconazole**
Candida albicans ATCC 90028	1250	19.531
Candida krusei ATCC 6258	1250	39.0925

Several studies have demonstrated that secondary metabolites produced by soil bacteria can serve as weapons in microbial warfare, providing an advantage to producer strains when competing against other microbial competitors in the same ecological niche (26).

Biofilm eradication assay of bioactive metabolite

Antibiofilm studies demonstrated a dose-dependent activity. The antibiofilm activity of bioactive metabolite extract was examined by minimum biofilm eradication concentration (MBEC) assay. The antibiofilm value of bioactive metabolite extract was determined 1250 µg/mL for *Staphylococcus aureus*, 1250 µg/mL for *Staphylococcus epidermidis* and 1250 µg/mL for *Candida albicans*. It means, the extract showed the same efficiency to selected microorganisms and eradicate their biofilm structure at the same dose. 1250 µg/mL of bioactive metabolite extract is a proper concentration for eradicating the biofilm formation of tested microorganisms.

Brine shrimp lethality test of bioactive metabolite

The brine shrimp lethality test was determined at 2500, 1250, 625, 312, and 156 µg/mL concentrations. The lethality values of *Artemia salina* larvae are shown in Table 4. 2500 and 1250 µg/mL concentrations are the most effective doses on larvae. The larvae are counted out of 10 larvae.

Table 4. Results of the Brine shrimp lethality test.

Bioactive Metabolite Concentrations (µg/mL)	**Amount of Alive *Artemia salina* Larvae (out of 10)**	**Percentage (%) Effect of Bioactive Metabolite**
2500	0	100%
1250	2	80%
625	3 ± 1	60-70%
312	3 ± 1	60-70%
156	4 ± 1	50-60%

The cytotoxic efficacy of the bioactive metabolite extract is significantly observed at different concentrations. It can be used as an anticancer drug in cell culture studies. The studies will be continued to understand all of the efficacy mechanisms with different tests.

CONCLUSION

The search for novel microorganism species from soils has gained momentum in recent years. Nature acts as a prominent reservoir for isolating the microorganisms that have various biological activities. The results from in vitro experiments, a *Brevibacterium spp.* called *Brevibacterium frigoritolerans* was isolated. In this study, many biological activities of this isolate have been tested and proved. The isolated novel bacterial strain was characterized by morphological, molecular, antimicrobial resistance, and chromium tolerance features and activities. For the determination of *Brevibacterium spp.* isolates antibiotic resistance, AmC-30 (amoxicilin+clavulanic acid 30/10 µg), SAM-20 (ampicillin+sulbactam 10/10 µg), IPM-10 (imipenem 10 µg), B (bacitracin 0.004 µg), OFX-10 (ofloxacin 10 µg) and TEC-30 (teicoplanin 30 µg) standard antibiotic discs were used. IPM-10 (imipenem 10 µg) was the most effective and B (bacitracin 0.004 µg) is not effective at all. Chromium is a heavy metal and causes environmental pollution. It accumulates in soil and is a very carcinogenic substance. For this reason, the chromium tolerance of *Brevibacterium spp.* isolates were determined. Das and Mishra showed that *Brevibacterium spp.* tolerates to potassium chromate (K_2CrO_4) (27). This research proved that

Brevibacterium spp. isolates tolerate both potassium chromate (K_2CrO_4) and chromium(III) chloride ($CrCl_3$) salts. The crude extract was checked for antibacterial, antibiofilm, and cytotoxic activities to identify the mechanisms of bioactive metabolite. *Brevibacterium spp*. showed different bioactive features, which highlighted its importance as potential pharmacological agents. Hence, there could be a probability of a new bioactive compound in the crude extract, which might provide a basis for further development of novel compounds from *Brevibacterium spp*. In Oskay's research, bioactive metabolites of Actinobacteria showed antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* microorganisms (28). In the present study, bioactive metabolites of *Brevibacterium spp*. isolates that member of Actinobacteria showed antimicrobial activity against *Staphylococcus aureus* ATCC29213, *Escherichia coli* ATCC 35218, *Klebsiella pneumonia* ATCC 700603, *Bacillus subtilis* NRRL B478, *Staphylococcus epidermidis* ATCC 14990*, Streptococcus pyogenes* ATCC 13615 *Listeria monocytogenes* ATCC 19111, *Candida albicans* ATCC 90028*, Candida krusei* ATCC 6258 and antibiofilm activity against *Staphylococcus aureus* ATCC29213, *Staphylococcus epidermidis* ATCC 14990*, Candida albicans* ATCC 90028 at different concentrations of the crude extract. It has been previously shown that bioactive metabolites of Actinobacteria at 0.1-10 µg/mL concentrations showed a 100% cytotoxic activity on *Artemia salina* larvae (29). In the current study, 80-100% cytotoxic activities at 2500 and 1250 µg/mL concentrations were determined. There are limited data in the literature about the activities of *Brevibacterium* bioactive metabolites. This research also provided new insight into the development of good candidates for pharmaceutical and bioactive natural products. The development of new drugs can be a candidate for curing several types of diseases (30). The current attempt of using *Brevibacterium* isolates and its bioactive metabolites will be favorable for antimicrobial, antitumoral, antibiofilm and cytotoxic activity tests.

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